REGULAR ARTICLE

blood advances

Antigen-specific immunotherapy with apitopes suppresses generation of FVIII inhibitor antibodies in HLA-transgenic mice

Katrien Pletinckx,[1] Kirsty S. Nicolson,[1,2] Heather B. Streeter,[2,3] William J. Sanderson,[1,2] Evelien Schurgers,[1] Lotta Jansson,[1] and David C. Wraith,[1-3]

[1]Apitope International NV, Diepenbeek, Belgium; [2]School of Cellular and Molecular Medicine, University of Bristol, Bristol, United Kingdom; and [3]Institute of Immunology and Immunotherapy, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom

Key Points

- ATX-F8-117 induces tolerance toward FVIII, thereby suppressing neutralizing antibody formation.
- ATX-F8-117 has the potential to treat inhibitor formation in susceptible patients with HA.

Hemophilia A (HA) is a blood clotting disorder that is caused by various genetic deficiencies in the factor VIII (FVIII)-encoding *F8* gene. Patients receiving FVIII-replacement therapy are at risk for developing neutralizing antibodies (FVIII inhibitors), rendering the FVIII-replacement therapy ineffective. Immunological tolerance toward FVIII can be achieved through immune tolerance induction protocols in some patients, but this is a lengthy and costly desensitization program. Long-term eradication of inhibitors in patients with HA could be achieved by antigen-specific immunotherapy targeting $CD4^+$ T-cells, because formation of FVIII inhibitors is T-cell dependent. Here, we report a peptide-based antigen-specific immunotherapy that is designed to specifically reestablish immune tolerance to FVIII through the development of antigen-processing-independent epitopes (apitopes). We identified 2 FVIII immunodominant peptides in immunized HLA-DRA*0101/DRB1*1501 transgenic (HLA-DR2tg) mice that were optimized for tolerogenicity. These modified peptide analogs were initially screened for recognition using FVIII-specific T-cell hybridoma clones from FVIII-immunized HLA-DR2tg mice. The FVIII apitopes were promiscuous and bound common human HLA-DRB1* allelic variants. The combination of these 2 FVIII apitopes (ATX-F8-117), administered according to a dose-escalation protocol, promoted T-cell tolerance toward FVIII in HLA-DR2tg mice. Furthermore, treatment with ATX-F8-117 significantly reduced FVIII inhibitor formation. ATX-F8-117 regulates anti-FVIII T-cell and B-cell responses, specifically the generation of FVIII inhibitors, revealing peptide-based antigen-specific immunotherapy as a promising approach to suppress and treat inhibitor formation in susceptible patients with HA.

Introduction

Hemophilia A (HA) is an X-linked inherited genetic disorder that results from a deficiency in the *F8* gene encoding the blood clotting factor VIII (FVIII).[1] Standard treatment of patients with HA involves life-long replacement therapy with human plasma-derived or recombinant FVIII (rFVIII) concentrates. However, ~30% of patients with HA develop neutralizing antibodies (FVIII inhibitors) that block the procoagulant function of the infused FVIII.[2] Long-term success in eradicating FVIII inhibitors can be achieved with immune tolerance induction therapy; however, this places a heavy burden on individual patients and health resources,[3] and alternative strategies are highly desirable.

Submitted 5 February 2021; accepted 7 July 2021; prepublished online on *Blood Advances* First Edition 16 September 2021; final version published online 29 March 2022. DOI 10.1182/bloodadvances.2021004451.

Data sharing requests should be sent to David C. Wraith (d.wraith@bham.ac.uk).

The full-text version of this article contains a data supplement.

The generation of FVIII inhibitors is $CD4^+$ T-cell dependent in murine hemophilia models[4-6] and in patients with HA.[7] FVIII-specific immune activation occurs through antigen-presenting cells (APCs) that internalize the FVIII protein and process and present antigenic peptides (epitopes) on major histocompatibility complex class II (MHCII) molecules to $CD4^+$ T-cells in the presence of costimulatory signals.[8] Studies have focused on the prevention of FVIII inhibitor formation by decreasing the immunogenicity of the FVIII protein following the removal of promiscuous T-cell and/or B-cell epitopes, without affecting FVIII coagulant activity.[9-11] Other experimental approaches induce FVIII immune tolerance by using broadly acting immunosuppressive drugs,[12] costimulatory pathway modulators,[13] agents to selectively deplete B-cell subsets,[14] antigen-coupled splenocytes,[15] nanoparticles with encapsulated antigen and rapamycin,[16] and others.[17] Clearly, antigen-specific strategies that actively reinstate long-lasting immune tolerance to FVIII while maintaining the immunosurveillance and antimicrobial immune responsiveness of patients with HA will have major therapeutic benefit.

Peptide immunotherapy using soluble $CD4^+$ T-cell epitopes has been successfully used for the promotion of immune tolerance in experimental models,[18] as well as in clinical trials of hypersensitivity reactions[19,20] and autoimmune disorders.[21-23] T-cell–tolerance mechanisms include deletion from the peripheral T-cell pool, anergy characterized by defective proliferation/interleukin-2 (IL-2) secretion, suppression through specialized regulatory T-cell (Treg) subsets, and immune deviation.[24] In the experimental autoimmune encephalomyelitis model of multiple sclerosis, administration of a peptide analog derived from myelin basic protein induces tolerance through the generation of $CD4^+$ T-cells with a regulatory phenotype.[25,26] Such induced $CD4^+$ regulatory type 1 cells were anergic, produced the anti-inflammatory cytokine IL-10,[27] suppressed naive T-cell activation in vivo,[28] and lacked the expression of the transcription factor FoxP3.[29,30] Chronic antigen encounter leads to transcriptional reprogramming within the cognate $CD4^+$ T-cell dictating anergy and the regulatory signature.[31] Crucial for the generation of the IL-10^+ $CD4^+$ Tregs is the mapping of the immunodominant T-cell epitopes with a high-affinity HLA-binding strength.[32] By escalating the peptide dose, subcutaneous delivery of antigen induces effective and safe $CD4^+$ T-cell tolerance, even at high doses,[33] a strategy that is also applied in allergen-specific immunotherapy[34] and in our recent clinical trials.[22,23,35] Despite compelling evidence that peptide immunotherapy shows great promise for establishing immunological tolerance,[36] it remains to be determined whether T-cell epitope desensitization can be an effective means to block immune activation to human recombinant protein therapeutics and plasma-derived proteins.

We previously demonstrated that T-cell epitopes able to induce $CD4^+$ T-cell tolerance in vivo mimic naturally processed antigen but bind MHCII complexes on APCs without the need for antigen processing.[37] These antigen-processing–independent epitopes (apitopes) can be identified in vitro using APCs lacking antigen-processing functionality through chemical fixation.[35] Numerous T-cell epitopes in human FVIII have been described[9,38-44]; however, our approach is to identify the FVIII apitopes from immunodominant T-cell epitopes mapped using hemophiliac mice humanized for human MHCII (HLA) molecules and T-cell hybridoma libraries. In this study, we use humanized HLA-DRA*0101/DRB1*1501transgenic (HLA-DR2tg) mice on a knockout background of murine MHCII molecules[45] given that, in addition to genetic and environmental factors, the HLA allele DRB1*1501 is reported to be a risk factor for the development of FVIII inhibitors.[46-48]

We designed 2 peptide analogs from human FVIII (hFVIII) immunodominant T-cell epitopes that mimicked the naturally processed form of the antigen but did not require antigen processing for presentation on human HLA-DR2. A combination of the FVIII apitopes, ATX-F8-117, promoted FVIII-specific T-cell tolerance in vivo and significantly suppressed FVIII inhibitor formation in a murine antibody model, demonstrating their potential for immune intervention in FVIII inhibitor–positive patients with HA.

Materials and methods

Mice

Mice expressing the human HLA-DRA*0101/DRB1*1501 (HLA-DR2) allele were originally obtained from Lars Fugger[45] and backcrossed onto the IA-β–knockout C57BL/6 genetic background (H2-Aβ^0 mice) lacking mouse endogenous MHCII genes.[49] The mice were bred under specific pathogen–free conditions at Charles River UK and were used in accordance with the guidelines of the local authorities.

Antigens

HLA-DRB1*1501-binding 15-mer peptides from hFVIII[50] were predicted using the bioinformatics algorithms Propred,[51] SYFPEITHI,[52] and the Immune Epitope Database and Analysis Resource consensus tool.[53,54] For apitopes to induce tolerance, they must be soluble.[18,55] When needed, N-terminal and C-terminal tags composed of glycine (G) and lysine (K) were added to optimize the solubility of 15-mer peptides.

Peptides were synthesized using Fmoc chemistry with an N-terminal free amine and a C-terminal amide by GL Biochem (Shanghai) Ltd (Shanghai, China) and dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) for storage at −80°C. Alternatively, peptides P1 (KKGPRCLTRYYSSFVNMEGKK) and P17 (KKGDNIMVTFRNQASRPYGKK) were synthesized by PolyPeptide Laboratories France (Strasbourg, France) as a non–Good Manufacturing Process batch and stored as a stock solution in phosphate buffered saline (PBS; Lonza, Basel, Switzerland) at −80°C. Recombinant hFVIII (rhFVIII) (ADVATE; Baxter, Deerfield, IL) was purchased from the Bristol and South West Haemophilia Group, Bristol Haematology and Oncology Centre.

Mouse immunization and T-cell–proliferation assay

Mice were immunized subcutaneously at the base of the tail with 40 μg of rhFVIII or 100 μg of DNIMVTFRNQASRPY (DNIMV) and 100 μg of PRCLTRYYSSFVNME (PRCLT) emulsified in incomplete Freund adjuvant (Difco Laboratories, Detroit, MI) supplemented with 400 μg of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco). Ten days after immunization, draining lymph nodes and spleens were collected, and single-cell suspensions were prepared. Cells were seeded in triplicate, at 0.5×10^6 cells per well, in X-VIVO 15 media supplemented with 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (Lonza) in 96-well flat-bottom plates (Greiner Bio-One, Kremsmünster, Austria) with the indicated antigens. After 3 days, 60 μL of supernatant was harvested and stored at −20°C. Cells were pulsed with [^{3}H]-thymidine (Perkin Elmer, Waltham, MA), as described.[56] The cytokine content of culture supernatants was

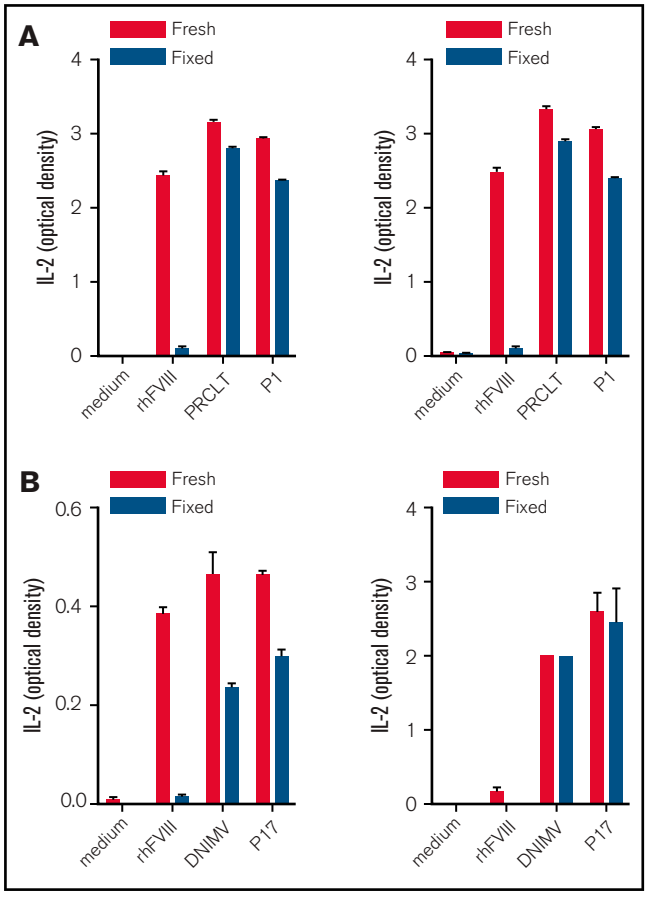

Figure 1. Peptides P1 and P17 are HLA-DRB1*1501–restricted FVIII apitopes. T-cell hybridoma clones derived from PRCLT- or DNIMV-immunized HLA-DR2tg mice were cocultured with an Epstein-Barr virus–transformed human HLA-DRB1*1501–expressing B cell line (MGAR). MGAR cells were left untreated (fresh) or were fixed with 0.5% paraformaldehyde (fixed) to prevent antigen processing. Recognition of peptides P1, P17, PRCLT, and DNIMV or rhFVIII by 2 clones specific for PRCLT (A) and 2 clones specific for DNIMV (B) was addressed after 48 hours by analyzing IL-2 cytokine secretion in the supernatants by ELISA.

analyzed with FlowCytomix Multiplex technology (eBioscience, San Diego, CA), according to the manufacturer's instructions. Fluorescence intensity was measured on a BD Accuri or a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

T-cell hybridoma cell culture

T-cell hybridomas were generated as described.[57] Briefly, lymph node cells or $CD4^+$ T-cells purified from mice immunized with rhFVIII (ADVATE) were cocultured in the presence of rhFVIII and irradiated (30 Gy) syngeneic splenocytes for 3 to 10 days at 37°C, 5% CO_2 in supplemented X-VIVO 15 media. Lymphoblasts were fused with TCRα-β- BW5147 fusion partner cells[58] using polyethylene glycol (Sigma-Aldrich). T-cell hybridoma growth was selected upon addition of hypoxanthine-aminopterin-thymidine medium (Sigma-Aldrich). Clones were tested for antigen specificity by culturing hybridoma cells with a lymphoblastoid cell line homozygous for HLA-DRB1*1501 (MGAR; International Histocompatibility Working Group, Seattle, WA) as APCs and 10 to 20 μg/mL antigen. After 48 hours, IL-2 production in supernatants was assessed by enzyme-linked immunosorbent assay (ELISA; eBioscience). An antigen-presentation assay was performed by coculturing 5×10^4 hybridoma clones with 5×10^4 MGAR cells, which were either left untreated or previously fixed with 0.5% paraformaldehyde (Sigma-Aldrich), in the presence of antigens for 48 hours, followed by analysis of the IL-2 cytokine content of supernatant.[59]

MHCII peptide binding competition assay

Relative affinity binding of peptides to common human HLA-DRB1 molecules was determined using ProImmune's REVEAL in vitro HLA binding assay (ProImmune, Oxford, UK).

Human PBMC assay

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors (NHS National Blood Service, Bristol, UK) or from patients with congenital HA, with a current negative FVIII inhibitor status, at the Haemophilia Treatment Center at Radboud University Medical Center (Nijmegen, The Netherlands), as previously described.[60] All studies were performed with the approval of the local ethics committees and after informed consent was obtained from the patients and healthy volunteers. PBMCs were stimulated with 5 to 10 μg/mL rhFVIII, 20 to 40 μg/mL peptides, or 20 μg/mL tuberculin purified protein derivative (PPD) (Statens Serum Institute, Copenhagen, Denmark) as a positive control, and proliferation was measured as previously described.[35]

FVIII antibody model

HLA-DR2tg mice (FVIII sufficient) were prophylactically treated subcutaneously with equivalent doses of peptides P1 and P17 (ATX-F8-117) by dose escalation (0.1-1-10-100-100-100 μg of each peptide) in 100 μL of PBS for the 2 weeks preceding immunization. Control animals were treated with a DR2-binding prostatic acid phosphatase (PAP) 133-152 control peptide[61] in 100 μL of PBS. Animals were immunized subcutaneously weekly with 1 μg of rhFVIII in 100 μL of sterile water for injection, as provided by the manufacturer. Plasma samples were taken biweekly and collected by mixing whole blood in sodium citrate buffer (Merck, Darmstadt, Germany) followed by a 10-minute centrifugation at 2500*g*. For a therapeutic treatment regimen, immunization with rhFVIII was initiated before the start of peptide treatment with ATX-F8-117 by dose escalation (0.1-1-10-100-100 μg of each peptide). Detailed descriptions of the numbers of mice, timing of peptide administration, and FVIII immunization and bleeding are provided in Figures 3 and 4.

Anti-FVIII antibody titer determination

Ninety-six well half-area plates (Corning) were coated overnight with 1 μg/mL rhFVIII in carbonate coating buffer at 4°C. Plates were blocked with 10% fetal bovine serum (Life Technologies, Carlsbad, CA) in PBS. Plasma samples were loaded as serial dilutions in 10% fetal bovine serum/PBS. Anti-FVIII immunoglobulin G (IgG) antibodies were detected using horseradish peroxidase–conjugated anti-mouse IgG (Abcam, Cambridge, UK), followed by 3,3',5,5'-tetramethylbenzidine substrate solution (Thermo Fisher Scientific, Waltham, MA). Absorbance was read at 450 nm using an ELISA reader (Tecan, Maennedorf, Switzerland). Antibody titer was expressed as the highest dilution of plasma sample showing a positive result (optical density > cutoff point). The cutoff point was set at 10× average of blank controls analyzed in 1 assay. Alternatively, the concentration of anti-FVIII–specific antibodies was estimated from a standard curve

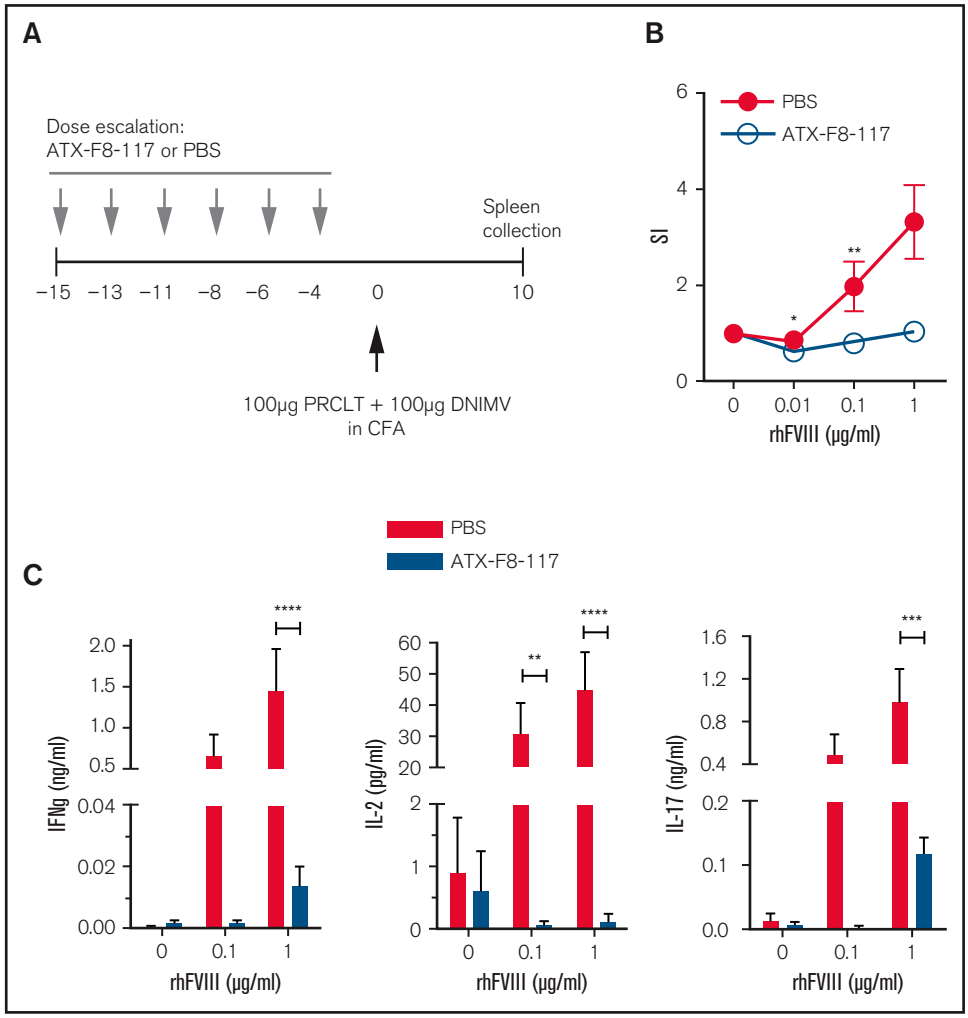

Figure 2. ATX-F8-117 induces FVIII-specific T-cell unresponsiveness in humanized HLA-DR2tg mice. (A) Groups of 8 to 10 HLA-DR2tg mice (FVIII$^{+/+}$) were pretreated with a combination of peptides P1 and P17 (ATX-F8-117), according to the dose-escalation scheme (0.1-1-10-100-100-100µg of each peptide), or PBS. Following pretreatment, mice were immunized with peptides PRCLT and DNIMV in complete Freund adjuvant (CFA) containing 400 µg of heat-killed *M tuberculosis*. (B) T-cell activation was assessed 10 days after the immunization by determining the proliferation of splenocytes upon restimulation with rhFVIII. Data are mean ± standard error of the mean (SEM). *$P < .05$, **$P < .01$, repeated-measures 2-way analysis of variance (ANOVA) of log-transformed data. (C) Cytokine content of the cell culture supernatants restimulated with rhFVIII was analyzed using cytokine bead array technology. Data are mean ± SEM. **$P < .01$, ***$P < .001$, ****$P < .0001$, repeated-measures 2-way ANOVA and Bonferroni post test. IFN-g, interferon-γ; SI, stimulation index.

obtained using serial dilutions of anti-human FVIII IgG2a (Thermo Fisher Scientific), starting at 50 ng/mL.

Anti-FVIII IgG isotype ELISA

IgG subclass of anti-FVIII antibodies was assessed using an in-house established ELISA assay. Briefly, 96-well plates (Corning) were coated overnight with 1 µg/mL rhFVIII in PBS at room temperature. Plates were blocked using 1% bovine serum albumin (Sigma-Aldrich) in PBS. Standards or plasma samples were diluted in 1% bovine serum albumin in PBS. Anti-FVIII IgG isotype-specific antibodies were detected by anti-mouse horseradish peroxidase–conjugated IgG1, IgG2a (both from Southern Biotech, Birmingham, AL), IgG2b, or IgG2c (both from Abcam) and 3,3',5,5'-tetramethylbenzidine substrate solution (Perbio Science). Absorbance was detected at 450 nm using an ELISA reader (Tecan). The concentration of anti-FVIII isotype–specific antibodies was estimated from a standard curve obtained using serial dilutions of mouse anti-human FVIII IgG1 (Merck Millipore), anti-human FVIII IgG2a (Thermo Fisher Scientific) starting at 50 ng/mL, or mouse anti-FVIII reference plasma starting at 50 arbitrary units per milliliter.

FVIII inhibitor assay

Anti-FVIII inhibitor antibodies were determined by a chromogenic method using a Chromogenix Coatest SP4 Factor VIII chromogenic assay (Diapharma, West Chester, OH). Briefly, plasma samples were inactivated by incubation at 56°C for 30 minutes and mixed with human pooled plasma (1 IU/mL FVIII) at a 1:1 ratio for incubation at 2 hours at 37°C, after which the manufacturer's instructions were followed. The percentage of residual FVIII activity in the test sample was calculated compared with the reference samples, and FVIII inhibitor titers were expressed in Bethesda Units (BU)/mL.

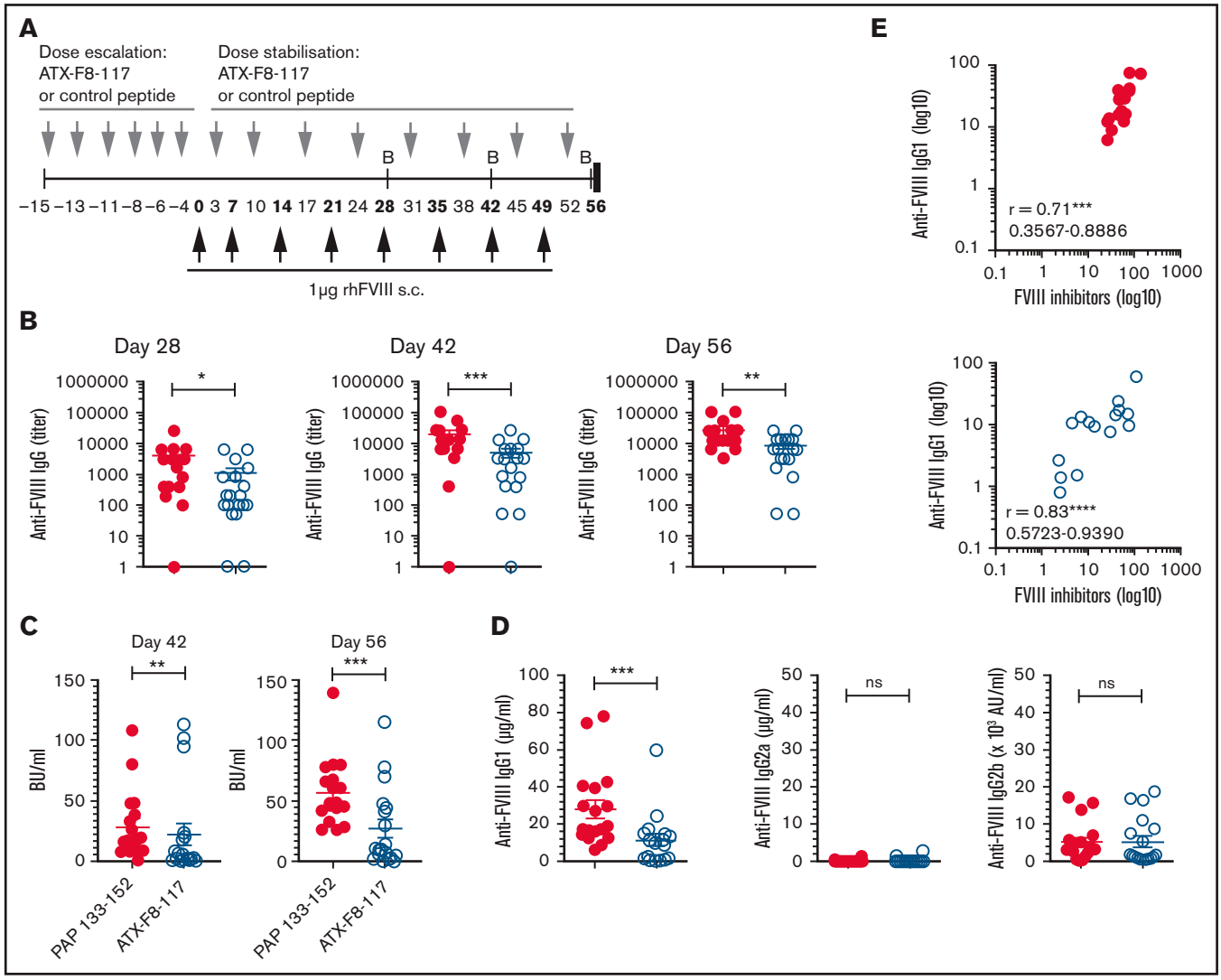

Figure 3. Preventive treatment with ATX-F8-117 reduces FVIII inhibitor formation in an FVIII antibody model. (A) Groups of 18 or 19 HLA-DR2tg mice (FVIII$^{+/+}$) were treated by dose escalation with ATX-F8-117 or PAP 133-152 as a peptide control. Four days after the peptide pretreatment, mice were primed 8 times, at weekly intervals, via subcutaneous (s.c.) flank injections with 1 µg of rhFVIII. Treatment with ATX-F8-117 or PAP 133-152 control was continued once weekly for 8 additional weeks starting 3 days after the initial FVIII priming. Plasma samples were collected from both treatment groups as indicated (B = bleed). (B) Plasma was collected at days 28, 42, and 56, and total anti-FVIII IgG levels were determined by ELISA. Graphs show end point anti-FVIII IgG titers on a log-scale; data are mean ± standard error of the mean (SEM). Each circle represents 1 mouse. Open blue circles, ATX-F8-117; filled red circles, PAP 133-152. (C) FVIII inhibitors from plasma collected at the indicated time points were analyzed using a modified Bethesda assay. Data are mean ± SEM; each circle represents 1 mouse. (D) Plasma was collected at day 56, and anti-FVIII IgG subclass distribution was determined by ELISA. Data are mean ± SEM; each circle represents 1 mouse. *$P < .05$, **$P < .01$, ***$P < .001$, 2-tailed Mann-Whitney U test (B-D). (E) Correlation between anti-FVIII IgG1 subclass antibodies and FVIII inhibitors in plasma samples collected at day 56 from animals treated with PAP 133-152 (upper panel) or ATX-F8-117 (lower panel) using 2-tailed nonparametric Spearman correlation analysis (r); 95% confidence intervals are also shown. Data are representative of 2 experiments performed. ***$P < .001$, ****$P < .0001$. ns, not significant; Open blue circles, ATX-F8-117; filled red circles, PAP 133-152.

Statistics

Statistical significance was determined using GraphPad Prism software.

Results

Identification of 2 major human FVIII T-cell apitopes (P1 and P17) using humanized HLA-DR2 mice

A set of 15-mer peptides of hFVIII was selected (Table 1) based on the core residues of high-affinity HLA-DRB1*1501 binding peptides predicted by multiple binding-prediction algorithms. The immunodominant peptides were identified by T-cell hybridoma technology using rhFVIII-immunized FVIII-sufficient (FVIII$^{+/+}$) mice expressing the human HLA-DR2 molecule. T-cell hybridomas were derived from mice immunized with rhFVIII (ADVATE); all responded to the FVIII protein. The majority of T-cell hybridoma clones responded to the peptides with the sequence DNIMV and PRCLT (40.7% and 11.1%, respectively). T-cell hybridoma clones were also produced from FVIII$^{-/-}$ HLA-DR2tg mice (supplemental Table 1) with similar results. Overall, these data indicate that these 2 peptides constitute 2 immunodominant T-cell epitopes in the HLA-DR2–restricted CD4^{+} T-cell

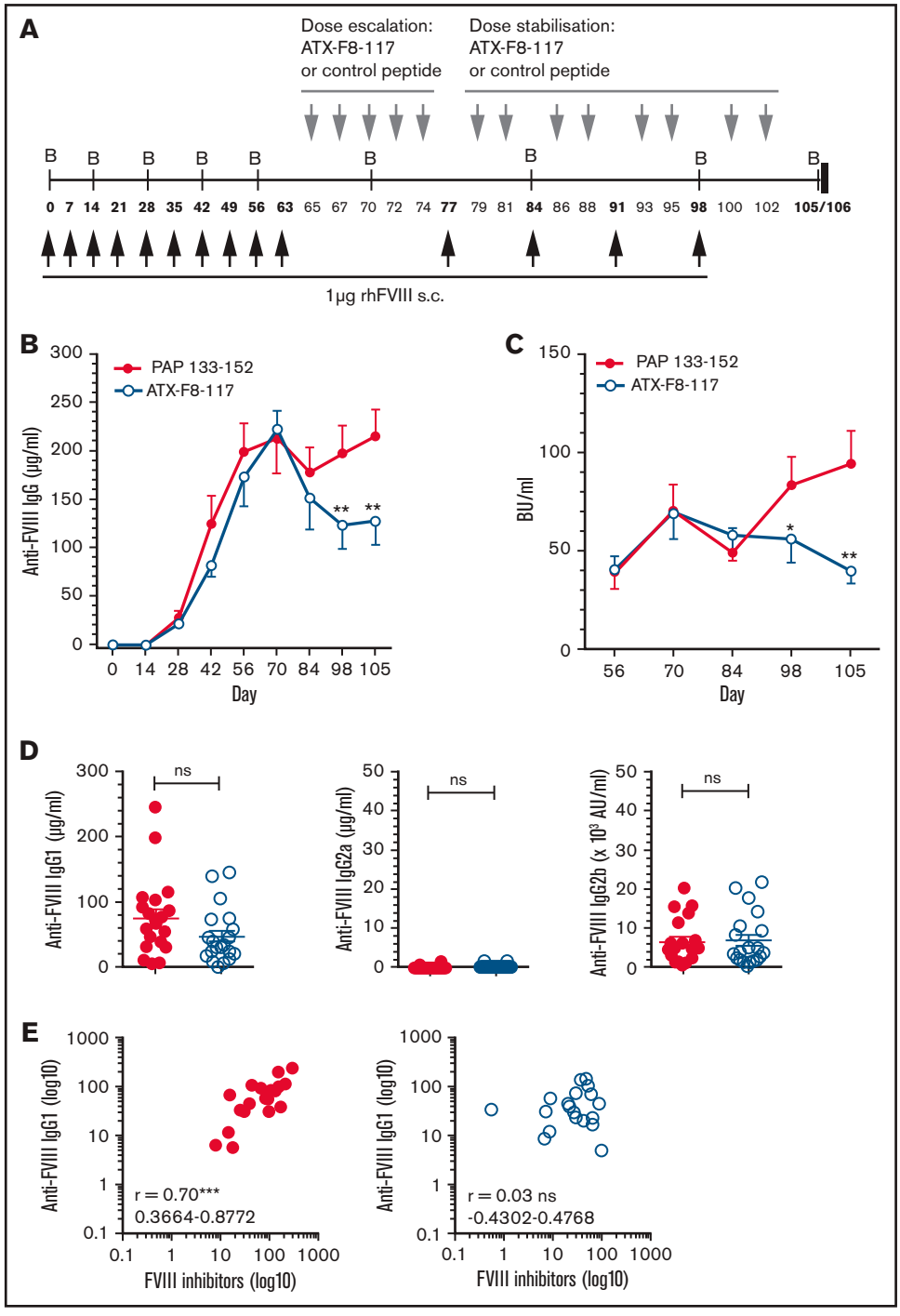

Figure 4. Therapeutic application of ATX-F8-117 reduces the formation of new FVIII inhibitor antibodies in a primed FVIII antibody model. (A) Groups of 20 HLA-DR2tg mice (FVIII$^{+/+}$) were primed with 10 weekly immunizations of rhFVIII, followed by dose-escalation treatment with ATX-F8-117 or PAP 133-152 via subcutaneous flank injections. Three days after the dose-escalating peptide treatment, mice were primed 4 times, at weekly intervals, via subcutaneous (s.c.) flank injections with 1 µg of rhFVIII. Treatment with ATX-F8-117 or PAP 133-152 control was continued twice weekly starting 2 days after FVIII priming. Plasma samples were collected from both treatment groups as indicated (B = bleed). (B) Plasma was collected at the indicated time points, and total anti-FVIII IgG levels were determined by ELISA. Data are mean ± standard error of the mean (SEM). (C) FVIII inhibitors from plasma collected at the indicated time points were analyzed using a modified Bethesda assay. Data are mean ± SEM. (D) Plasma was collected at day 105-106, and anti-FVIII IgG subclass distribution was determined by ELISA. Each circle represents 1 mouse; data are mean ± SEM. Open blue circles, ATX-F8-117; filled red circles, PAP 133-152.*$P < .05$, **$P < .01$, robust regression model using M estimation, Huber weighting, and the default parameter c = 1.345 with treatment as a factor and day 56 data as a covariate (B-C). (E) Correlation between anti-FVIII IgG1 subclass antibodies and FVIII inhibitors in plasma samples collected at day 105-106 from animal treated with PAP 133-152 (left panel) or ATX-F8-117 (right panel) using 2-tailed nonparametric Spearman correlation analysis (r); 95% confidence intervals are also shown. ***$P < .001$. ns, not significant.

Table 1. Human FVIII HLA-DRB1*1501–restricted T-cell–binding epitopes

Epitope sequence	At position
TQTLHKFILLFAVFD	208-222
MHTVNGYVNRSLPGL	250-264
LGQFLLFCHISSHQH	322-336
DTLLIIFKNQASRPY	478-492
PRCLTRYYSSFVNME	545-559
TENIQRFLPNPAGVQ	607-621
DNIMVTFRNQASRPY	1788-1802
SLYISQFIIMYSLDG	2114-2128
GTLMVFFGNVDSSGI	2140-2154
PPIIARYIRLHPTHY	2161-2175
PPLLTRYLRIHPQSW	2318-2332
RYLRIHPQSWVHQIA	2322-2336

Fifteen-mer high-affinity binding MHCII epitopes of the hFVIII protein were predicted for human HLA-DRB1*1501 alleles using bioinformatics tools. Peptides with amino acids highlighted in bold are designated by the first 5 N-terminal amino acids in their sequence.

response to hFVIII in HLA-DR2tg mice, confirming the data of Steinitz et al,[38] who identified T-cell epitopes using a similar set-up; 2 of their epitopes overlap with our peptides.

Peptide characteristics of PRCLT and DNIMV were optimized for solubility[55] by adding N-terminal and C-terminal tags composed of the linker glycine (G) and charged amino acid lysine (K) outside of the core binding residues to generate the modified peptides P1 (KKG-PRCLT-GKK) and P17 (KKG-DNIMV-GKK). We previously demonstrated that therapeutic peptides should mimic the HLA binding of the naturally processed epitopes of the target antigen by acting as apitopes.[37] Therefore, we addressed whether FVIII-specific T-cell hybridoma clones from HLA-DR2tg mice responded to the modified peptides P1 and P17 on paraformaldehyde-fixed APCs. PRCLT-specific clones produced IL-2 in response to PRCLT and the modified P1 peptide on fresh and fixed APCs, but recognized FVIII on fresh APCs only (Figure 1A). Similarly, DNIMV-specific clones were activated by incubation with peptides P17 or DNIMV on fresh and fixed APCs (Figure 1B), whereas no IL-2 was measured in the presence of FVIII and fixed APCs.

In sum, peptides P1 and P17 were designed from the human FVIII immunodominant HLA-DR2 binding peptides PRCLT and DNIMV, respectively, and behave as apitopes.

Human FVIII apitopes P1 and P17 are HLA pan-DR binding peptides

Because apitopes P1 and P17 were identified using T-cell clones from HLA-DR2tg mice, an in vitro major histocompatibility complex (MHC) binding competition assay was performed to study the apitope binding affinities to common human HLA-DRB1 allelic variants. The ProImmune REVEAL binding assay detects peptide binding to recombinant MHCII molecules based on peptide-induced stabilization of the MHC molecule. Apitopes P1 and P17 bind with a higher relative affinity to HLA-DRA*0101; DRB1*15:01 complexes than the control peptide, as expected (50% inhibitory concentration [IC_{50}] < 0.1 μM; Table 2). Interestingly, P1 and P17 peptides bind HLA-DRA*0101; DRB1*11:01 and HLA-DRA*0101; DRB1*04:01 complexes with intermediate to high efficiency compared with the control peptide (Table 2), suggesting that P1 and P17 bind common human HLA-DRB1 molecules. Of note, because a different assay is performed for every HLA-DRB1 molecule, IC_{50} values can only be compared between the 2 peptides tested for 1 particular HLA-DRB1 molecule. Hence, the results of this assay can only be relative. IC_{50} values cannot be compared among different HLA-DRB1 molecules.

Next, we addressed whether P1 and P17 induced proliferation in PBMCs from healthy subjects ($FVIII^{+/+}$) and patients with HA ($FVIII^{-/-}$) expressing a variety of HLA-DRB1* allelic variants. PBMCs from healthy individuals commonly recognized rFVIII and $\geq$1 apitope. Interestingly, PBMCs from 14 of 23 patients with HA with a current negative inhibitor status responded to rFVIII by proliferation, and 9 of 14 recognized 1 or both apitopes, whereas 9 of 23 patients with HA did not respond to rFVIII at all (Table 3; supplemental Table 2). Overall, this implies that P1 and P17 are major hFVIII epitopes in patients with HA, and healthy donors and patients with HA expressing various HLA haplotypes frequently respond to the peptides.

Peptide cocktail ATX-F8-117 induces tolerance among FVIII-specific T-cells

To determine whether P1 and P17 were capable of regulating T-cell responses to hFVIII, HLA-DR2tg mice were treated with a combination of P1 and P17 (ATX-F8-117), according to a dose-escalation strategy,[33] followed by immunization (Figure 2A).

Spleens from mice that received control treatment with PBS proliferated vigorously upon recall with rhFVIII in vitro (Figure 2B). However, subcutaneous treatment with ATX-F8-117 completely abrogated the proliferative response of splenocyte cultures upon rechallenge with rhFVIII (Figure 2B). Of note, splenocytes from both treatment groups showed a comparable recall response to PPD as an immunization control (supplemental Figure 1). Furthermore, spleens from mice treated with PBS secreted high levels of interferon-γ, IL-2, and IL-17 in response to antigen restimulation in vitro (Figure 2C). Repetitive treatment with ATX-F8-117 significantly reduced splenic cytokine production of interferon-γ, IL-2, and IL-17 when restimulated with rhFVIII. Together, ATX-F8-117 induces antigen-specific T-cell tolerance upon subcutaneous administration in humanized HLA-DR2tg mice.

When performing the same experiment in $FVIII^{-/-}$ HLA-DR2tg mice, a reduction in the proliferative response toward rhFVIII was observed in splenocyte cultures from ATX-F8-117–treated mice compared with controls (supplemental Figure 2).

Table 2. Apitopes P1 and P17 bind common human HLA-DRB1* molecules

	HLA-DRA*01:01; DRB1*15:01	HLA-DRA*01:01; DRB1*03:01	HLA-DRA*01:01; DRB1*04:01	HLA-DRA*01:01; DRB1*11:01
P1	0.06	32.21	0.40	1.28
P17	0.04	21.90	5.09	4.19

Data are IC_{50} (μM).

Affinity binding of apitopes P1 and P17 to the indicated MHC alleles, as determined using an in vitro MHC binding assay (ProImmune REVEAL assay) studying displacement of a control peptide by apitopes P1 and P17.

Table 3. Apitopes P1 and P17 induce proliferation in human PBMCs from healthy blood donors and patients with HA

	Response to rhFVIII and 1 or both apitopes	Response to 1 or both apitopes (FVIII responders)	No response to rhFVIII
$FVIII^{+/+}$ healthy donors	11/15 (73%)	9/11 (82%)	4/15 (27%)
$FVIII^{-/-}$ patients	14/23 (61%)	9/14 (64%)	9/23 (39%)

PBMCs isolated from peripheral blood of healthy donors or patients with HA were cultured with graded concentrations of rhFVIII or peptides P1 and P17. PBMC proliferation was assessed at day 6 to day 8 of culture by [^{3}H]-thymidine incorporation and expressed as the stimulation index (SI). A positive response to peptides P1 or P17 and rhFVIII was defined as SI $>$ 2.

ATX-F8-117 suppresses FVIII inhibitor formation in HLA-DR2tg mice

Because ATX-F8-117 can regulate the anti-FVIII T-cell response, we evaluated the efficacy of the peptide cocktail to modulate anti-FVIII humoral immune responses in a murine FVIII antibody model. As outlined in Figure 3A, HLA-DR2tg mice were pretreated with ATX-F8-117 or PAP 133-152 (control),[61] according to a dose-escalation regimen, followed by repeated immunization with rhFVIII. Peptide treatment was continued at the highest dose and administered 3 days after each rhFVIII immunization.

In both groups, mice developed detectable levels of anti-FVIII IgG antibodies after 4 subcutaneous injections with rhFVIII (day 28; Figure 3B); recurrent rhFVIII immunizations further elevated total anti-FVIII IgG titers up to day 56 in both groups (Figure 3B). However, pretreatment with ATX-F8-117 successfully decreased total anti-FVIII IgG levels compared with controls (Figure 3B). Similarly, HLA-DR2tg mice started to develop FVIII inhibitors after 4 subcutaneous immunizations with rhFVIII (day 28, data not shown); subsequent rhFVIII injections led to a strong increase in the FVIII inhibitor levels in control animals (to 56.6 BU/mL on day 56; Figure 3C). In contrast, pretreatment with ATX-F8-117 significantly reduced the FVIII inhibitor levels to 50% at day 56 (26.9 BU/mL). All levels of anti-FVIII-IgG and FVIII inhibitors for individual mice, can be found in supplemental Table 3. The IgG subclass distribution of anti-FVIII antibodies of control-treated animals was predominantly IgG1 (Figure 3D). Lower proportions belonged to the IgG2b subclass, and very low levels of anti-FVIII IgG2a isotype antibodies could be detected at day 56. Upon prophylactic treatment with ATX-F8-117, the anti-FVIII IgG1 subclass antibodies were significantly reduced compared with control mice (Figure 3D). Interestingly, the correlation between FVIII inhibitors and anti-FVIII IgG1 subclass antibodies, but not anti-FVIII IgG2a and IgG2b antibodies, reached statistical significance (Figure 3E, data not shown).

Thus, pretreatment with ATX-F8-117 is highly efficacious in suppressing FVIII inhibitor formation in a murine FVIII antibody model developing predominantly anti-FVIII IgG1 subclass antibodies.

ATX-F8-117 suppresses new FVIII inhibitor formation in HLA-DR2tg mice with an ongoing anti-FVIII immune response

To demonstrate the efficacy of ATX-F8-117 in modulating the anti-FVIII immune responses in animals with preexisting FVIII inhibitory antibodies, HLA-DR2tg mice were repetitively immunized subcutaneously with rhFVIII during which peptide treatment was initiated after the tenth immunization with rhFVIII (day 65), as outlined in Figure 4A.

Prior to the start of peptide therapy (day 65), all mice developed high levels of anti-FVIII IgG antibodies (day 56; Figure 4B). Following control treatment, plasma levels of anti-FVIII IgG antibodies increased with recurrent rhFVIII immunizations up to day 105-106 after a small initial decline in antibody levels due to a break in the rhFVIII immunization regimen at day 70 (Figure 4B). Therapeutic intervention with ATX-F8-117 significantly reduced total anti-FVIII IgG levels from day 98 onward compared with controls (Figure 4B). Similarly, mice developed high FVIII inhibitory antibodies prior to peptide treatment start (day 56; Figure 4C). FVIII inhibitors increased remarkably in the control group following repeated rhFVIII injections (Figure 4C) from 39.0 BU/mL on day 56 to 93.8 BU/mL on day 105-106. Treatment with ATX-F8-117 successfully reduced plasma levels of FVIII inhibitors up to 58% at day 105-106 (39.6 BU/mL) compared with control animals (Figure 4C). All levels of anti-FVIII-IgG and FVIII inhibitors for individual mice, can be found in supplemental Table 4.

Again, the IgG subclass distribution of anti-FVIII antibodies from control animals was predominantly IgG1 (Figure 4D). Also, fewer anti-FVIII antibodies belonged to the IgG2b subclass family, and no anti-FVIII IgG2a isotype antibodies could be detected in plasma from controls (Figure 4D). Upon therapeutic treatment with ATX-F8-117, lower anti-FVIII IgG1 subclass antibodies were detected compared with the control group (Figure 4D). Correlation analyses between FVIII inhibitors and the anti-FVIII IgG1 subclass antibodies reached statistical significance in the control group (Figure 4E, left panel).

These results show that therapeutic intervention with ATX-F8-117 is efficacious in preventing new FVIII inhibitor formation in HLA-DR2tg mice with circulating anti-FVIII inhibitory antibodies.

Discussion

There is a clear need for improved approaches for induction of immunological tolerance to FVIII in HA. Long-term success in eradicating FVIII inhibitors can be achieved with immune tolerance induction therapy. This involves the frequent administration of high doses of FVIII and is based on high dose tolerance that was first described by Mitchison in the 1960s.[62] Unfortunately, however, this approach is generally more effective in those with low levels of inhibitors and can fail in individuals with higher levels. Furthermore, the approach is highly expensive and requires frequent invasive treatment, thereby placing a heavy burden on individual patients and health resources[3]; alternative strategies are highly desirable. One such approach that has been tested in vitro involved transduction of human Tregs with cloned T-cell receptor genes from a patient with hemophilia's FVIII-specific T-cell.[63] The transduced cells suppressed T-cell proliferation in vitro and, most importantly, reduced antibody production by spleen cells from an immunized HLA-DR transgenic mouse. This provides evidence that Tregs can modulate the immune response to FVIII and also demonstrates that epitope-specific Tregs mediate linked suppression of the response to other epitopes within FVIII. This approach would be difficult to adopt for the general hemophiliac population because it would be highly patient specific or at least HLA-DR specific and would probably require frequent

infusions of Tregs. An alternative approach would be to promote the generation of a regulatory phenotype in vivo through the administration of pan-DR binding T-cell epitopes derived from FVIII. Our analysis reveals that 2 $CD4^+$ T-cell epitopes constitute the dominant HLA-DR2–restricted human FVIII epitopes in humanized $FVIII^{+/+}$ and hemophiliac transgenic mice. This confirms previous observations using similar technology, whereby Steinitz et al revealed that 2 of the 3 dominant epitopes (3 dominant epitopes identified from 8 immunogenic regions) overlap with the DNIMV and PRCTL peptides described here and showed that they bind common HLA-DRB1* molecules.[38] Apitopes P1 and P17 induced proliferation in PBMC cultures from healthy subjects and patients with HA expressing various HLA-DRB1* molecules. PBMCs from patients with HA responding to FVIII recognized ≥1 of the 2 apitopes. Furthermore, van Haren et al revealed that few core peptides were presented by HLA-DR molecules of FVIII-pulsed donor-derived human dendritic cells (DCs) with diverse HLA-DRB1* molecules.[64] Interestingly, 1 core peptide sequence FRNQASRPY, part of the P17 sequence, was a promiscuous pan-DR HLA-DR binding peptide. These data underscore the relevance and potential of the P1 and P17 apitopes for the treatment of inhibitor-positive patients with HA.

Apitopes P1 and P17 can bind MHCII molecules on APCs rendered incapable of antigen processing by chemical fixation (ie, they are antigen processing independent). Why is it important for peptides to be designed as apitopes? Tolerogenic peptides must be designed to mimic the naturally processed epitope to induce tolerance.[37] Recently, we have shown that apitopes selectively bind steady-state DCs in lymphoid organs and do not bind B cells or monocytes in vivo.[65] Empty or peptide-receptive MHCII molecules are abundantly expressed on the surface of steady-state and immature DCs, in particular,[66,67] and we know that these cells induce tolerance in vivo.[68]

Apitope cocktail ATX-F8-117 promoted antigen-specific T-cell tolerance in vivo, following a dose-escalation strategy, indicating that apitope immunotherapy is an effective means of tolerance promotion to protein therapeutics. The proliferative capacity and IL-2 production of $CD4^+$ T-cells were abrogated in splenocyte cultures restimulated in vitro with FVIII, but not with PPD, showing that apitope-mediated tolerance is antigen specific. In the absence of overt infection, immature and intermediate steady-state DC maturation stages are prevalent; these steady-state DCs present self-peptides for the induction of $CD4^+$ T-cell tolerance.[69]

Because ATX-F8-117 is designed to reinstate tolerance to FVIII replacement therapy, rather than to control bleeding episodes, in patients with HA, FVIII-sufficient HLA-DR2tg mice were used as an FVIII antibody model. The use of $FVIII^{+/+}$ mice as a model for FVIII inhibitor treatment reflects the clinical scenario of patients with HA, who have residual dysfunctional FVIII circulating but are at risk for developing immune responses to the replacement therapy. Reipert et al demonstrated that, although the rate of anti-FVIII titer development differs between $FVIII^{+/+}$ and FVIII-knockout animals, similar total anti-FVIII antibody levels occur after recurrent subcutaneous rhFVIII injections.[70] Here, HLA-DR2tg animals developed high levels of anti-FVIII IgG following 8 weekly subcutaneous immunizations with rhFVIII; nevertheless, the increase in inhibitor antibodies could still be reversed by apitope treatment.

Previous reports have shown that IgG1 and IgG4 were the most abundant IgG subclasses of anti-FVIII antibodies in patients with HA with inhibitors.[71] The occurrence of FVIII inhibitors in experimental hemophiliac mouse models can be attributed to the IgG1 and IgG2a subclasses, depending on the genetic background and immunization strategy.[70,72] In the model described here, HLA-DR2tg mice received recurrent rhFVIII immunizations in the absence of microbial stimuli that initiated predominantly T helper 2 cell–driven anti-FVIII IgG1 isotype development. Additionally, correlation analysis indicated that there was a direct association with FVIII inhibitors and IgG1 subclass antibodies in control animals. Hence, our data are consistent with the association between FVIII inhibitors and the IgG1 subclass described in hemophiliac mouse models and patients with HA.[70,71,73]

Data highlight the efficacy of ATX-F8-117 to suppress the formation of new FVIII inhibitors in naive and FVIII-primed HLA-DR2 humanized animals. A potential mode of action is believed to occur through the lack of the $CD4^+$ T-cell help required for B-cells to transform into an antibody-producing plasma cell. However, accumulating evidence indicates that Tregs can directly control B-cell antibody production in vitro.[74,75] Based on these data, it will be interesting to see how the anergic $CD4^+$ T-cells induced by ATX-F8-117 are able to control the anti-FVIII antibody response. Evidence that apitope treatment can reduce the levels of circulating antibodies in humans comes from a phase 1 clinical trial performed in patients with Graves' hyperthyroidism.[23]

Multiple inhibitory B-cell epitopes have been mapped to the A2 and C2 domains of the hFVIII protein, which are essential for its procoagulant activity; however, nonneutralizing antibodies may bind to multiple other FVIII surfaces, and some inhibitory antibodies also bind to other domains and may affect von Willebrand factor binding or FVIII clearance.[10] Interestingly, the FVIII immunodominant T-cell epitopes, as identified in this study, were mapped to the FVIII A2 (PRCLT) and A3 (DNIMV) domains, but not to the C2 domain, a finding that was confirmed elsewhere.[38] Peptide treatment with a single epitope can result in suppression of other epitopes within the same protein through linked suppression.[76,77] Interestingly, administration of each separate apitope (P1 or P17) in HLA-DR2tg mice, followed by immunization with full-length rhFVIII in complete Freund adjuvant, reduced cytokine production and proliferation in secondary lymphoid organs in response to rhFVIII restimulation ex vivo. Moreover, no anti-apitope antibodies were detected in the plasma of our experimental FVIII antibody model following rhFVIII immunization, dismissing the possibility that inhibitory FVIII antibodies decrease via direct absorption from the circulation upon apitope treatment. It should be noted that, although most FVIII B-cell epitopes are conformational epitopes,[10] screening patients with HA for the presence of anti-apitope antibodies prior to treatment with ATX-F8-117 remains vitally important to prevent peptide-induced anaphylaxis.[78] The promising results from recent clinical trials of apitope therapy in antibody-mediated Graves' disease support the clinical development of ATX-F8-117 for the suppression of inhibitor formation in people with HA and the use of apitopes for other anti-drug antibody complications.

Acknowledgments

The authors thank Terrie Walker-Smith, Lan Wang, Sarah Aerts, and Jason Caulfield for technical assistance; Richard Brammar for statistical analysis; and Christine Hough (Queen's University, Kingston, ON, Canada) for inhibitor assays.

Authorship

Contribution: K.P., L.J., H.B.S., E.S., and D.C.W. designed the study, interpreted data, and wrote the manuscript and K.P., K.S.N., H.B.S., and W.J.S. performed research and collected data.

Conflict-of-interest disclosure: K.P., K.S.N., W.J.S., E.S., and L.J. were employees of Apitope International NV at the time of this study. D.C.W. serves as Chief Scientific Officer for Apitope International NV on a consulting basis. H.B.S. declares no competing financial interests.

The current affiliation for K.S.N. is UCB Biopharma, Slough, United Kingdom.

ORCID profiles: D.C.W., 0000-0003-2147-5614; K.P., 0000-0002-4965-4887; W.J.S., 0000-0003-0170-9911; E.S., 0000-0001-8397-3112; L.J., 0000-0002-3434-9345.

Correspondence: David C. Wraith, Institute of Immunology and Immunotherapy, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom; e-mail: d.wraith@bham.ac.uk.

References

1. Hoyer LW. Hemophilia A. *N Engl J Med.* 1994;330(1):38-47.
2. Schep SJ, Schutgens REG, Fischer K, Boes ML. Review of immune tolerance induction in hemophilia A. *Blood Rev.* 2018;32(4):326-338.
3. Hay CR, DiMichele DM; International Immune Tolerance Study. The principal results of the International Immune Tolerance Study: a randomized dose comparison. *Blood.* 2012;119(6):1335-1344.
4. Waters B, Qadura M, Burnett E, et al. Anti-CD3 prevents factor VIII inhibitor development in hemophilia A mice by a regulatory $CD4^+CD25^+$-dependent mechanism and by shifting cytokine production to favor a Th1 response. *Blood.* 2009;113(1):193-203.
5. Qian J, Borovok M, Bi L, Kazazian HH Jr, Hoyer LW. Inhibitor antibody development and T cell response to human factor VIII in murine hemophilia A. *Thromb Haemost.* 1999;81(2):240-244.
6. Reipert BM, Sasgary M, Ahmad RU, Auer W, Turecek PL, Schwarz HP. Blockade of CD40/CD40 ligand interactions prevents induction of factor VIII inhibitors in hemophilic mice but does not induce lasting immune tolerance. *Thromb Haemost.* 2001;86(6):1345-1352.
7. Ragni MV, Bontempo FA, Lewis JH. Disappearance of inhibitor to factor VIII in HIV-infected hemophiliacs with progression to AIDS or severe ARC. *Transfusion.* 1989;29(5):447-449.
8. Wroblewska A, Reipert BM, Pratt KP, Voorberg J. Dangerous liaisons: how the immune system deals with factor VIII. *J Thromb Haemost.* 2013;11(1):47-55.
9. Jones TD, Phillips WJ, Smith BJ, et al. Identification and removal of a promiscuous $CD4^+$ T cell epitope from the C1 domain of factor VIII. *J Thromb Haemost.* 2005;3(5):991-1000.
10. Pratt KP, Thompson AR. B-cell and T-cell epitopes in anti-factor VIII immune responses. *Clin Rev Allergy Immunol.* 2009;37(2):80-95.
11. Kruse-Jarres R, St-Louis J, Greist A, et al. Efficacy and safety of OBI-1, an antihaemophilic factor VIII (recombinant), porcine sequence, in subjects with acquired haemophilia A. *Haemophilia.* 2015;21(2):162-170.
12. Moghimi B, Sack BK, Nayak S, Markusic DM, Mah CS, Herzog RW. Induction of tolerance to factor VIII by transient co-administration with rapamycin. *J Thromb Haemost.* 2011;9(8):1524-1533.
13. Hausl C, Ahmad RU, Schwarz HP, et al. Preventing restimulation of memory B cells in hemophilia A: a potential new strategy for the treatment of antibody-dependent immune disorders. *Blood.* 2004;104(1):115-122.
14. Zhang AH, Skupsky J, Scott DW. Effect of B-cell depletion using anti-CD20 therapy on inhibitory antibody formation to human FVIII in hemophilia A mice. *Blood.* 2011;117(7):2223-2226.
15. Adair P, Su Y, Scott DW. Tolerance induction in hemophilia A animal models: battling inhibitors with antigen-specific immunotherapies. *Discov Med.* 2013;15(84):275-282.
16. Maldonado RA, LaMothe RA, Ferrari JD, et al. Polymeric synthetic nanoparticles for the induction of antigen-specific immunological tolerance. *Proc Natl Acad Sci USA.* 2015;112(2):E156-E165.
17. Lacroix-Desmazes S, Voorberg J, Lillicrap D, Scott DW, Pratt KP. Tolerating factor VIII: recent progress. *Front Immunol.* 2020;10:2991.
18. Wraith DC. Designing antigens for the prevention and treatment of autoimmune diseases. *Curr Opin Chem Eng.* 2018;19:35-42.
19. Ellis AK, Frankish CW, O'Hehir RE, et al. Treatment with grass allergen peptides improves symptoms of grass pollen-induced allergic rhinoconjunctivitis. *J Allergy Clin Immunol.* 2017;140(2):486-496.
20. Goel G, King T, Daveson AJ, et al. Epitope-specific immunotherapy targeting CD4-positive T cells in coeliac disease: two randomised, double-blind, placebo-controlled phase 1 studies. *Lancet Gastroenterol Hepatol.* 2017;2(7):479-493.
21. Alhadj Ali M, Liu YF, Arif S, et al. Metabolic and immune effects of immunotherapy with proinsulin peptide in human new-onset type 1 diabetes. *Sci Transl Med.* 2017;9(402):eaaf7779.
22. Chataway J, Martin K, Barrell K, Sharrack B, Stolt P, Wraith DC; ATX-MS1467 Study Group. Effects of ATX-MS-1467 immunotherapy over 16 weeks in relapsing multiple sclerosis. *Neurology.* 2018;90(11):e955-e962.
23. Pearce SHS, Dayan C, Wraith DC, et al. Antigen-specific immunotherapy with thyrotropin receptor peptides in Graves' hyperthyroidism: a phase I study. *Thyroid.* 2019;29(7):1003-1011.

24. Walker LS, Abbas AK. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat Rev Immunol.* 2002;2(1):11-19.
25. Metzler B, Wraith DC. Inhibition of experimental autoimmune encephalomyelitis by inhalation but not oral administration of the encephalitogenic peptide: influence of MHC binding affinity. *Int Immunol.* 1993;5(9):1159-1165.
26. Liu GY, Wraith DC. Affinity for class II MHC determines the extent to which soluble peptides tolerize autoreactive T cells in naive and primed adult mice–implications for autoimmunity. *Int Immunol.* 1995;7(8):1255-1263.
27. Burkhart C, Liu GY, Anderton SM, Metzler B, Wraith DC. Peptide-induced T cell regulation of experimental autoimmune encephalomyelitis: a role for IL-10. *Int Immunol.* 1999;11(10):1625-1634.
28. Sundstedt A, O'Neill EJ, Nicolson KS, Wraith DC. Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo. *J Immunol.* 2003;170(3):1240-1248.
29. Vieira PL, Christensen JR, Minaee S, et al. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *J Immunol.* 2004;172(10):5986-5993.
30. Nicolson KS, O'Neill EJ, Sundstedt A, Streeter HB, Minaee S, Wraith DC. Antigen-induced IL-10+ regulatory T cells are independent of CD25+ regulatory cells for their growth, differentiation, and function. *J Immunol.* 2006;176(9):5329-5337.
31. Anderson PO, Manzo BA, Sundstedt A, et al. Persistent antigenic stimulation alters the transcription program in T cells, resulting in antigen-specific tolerance. *Eur J Immunol.* 2006;36(6):1374-1385.
32. Gabrysová L, Wraith DC. Antigenic strength controls the generation of antigen-specific IL-10-secreting T regulatory cells. *Eur J Immunol.* 2010;40(5): 1386-1395.
33. Burton BR, Britton GJ, Fang H, et al. Sequential transcriptional changes dictate safe and effective antigen-specific immunotherapy. *Nat Commun.* 2014;5(1):4741.
34. Larché M. T cell epitope-based allergy vaccines. *Curr Top Microbiol Immunol.* 2011;352:107-119.
35. Streeter HB, Rigden R, Martin KF, Scolding NJ, Wraith DC. Preclinical development and first-in-human study of ATX-MS-1467 for immunotherapy of MS. *Neurol Neuroimmunol Neuroinflamm.* 2015;2(3):e93.
36. Larché M, Wraith DC. Peptide-based therapeutic vaccines for allergic and autoimmune diseases. *Nat Med.* 2005;11(4 suppl):S69-S76.
37. Anderton SM, Viner NJ, Matharu P, Lowrey PA, Wraith DC. Influence of a dominant cryptic epitope on autoimmune T cell tolerance. *Nat Immunol.* 2002; 3(2):175-181.
38. Steinitz KN, van Helden PM, Binder B, et al. CD4+ T-cell epitopes associated with antibody responses after intravenously and subcutaneously applied human FVIII in humanized hemophilic E17 HLA-DRB1*1501 mice. *Blood.* 2012;119(17):4073-4082.
39. Hu GL, Okita DK, Conti-Fine BM. T cell recognition of the A2 domain of coagulation factor VIII in hemophilia patients and healthy subjects. *J Thromb Haemost.* 2004;2(11):1908-1917.
40. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Epitope repertoire of human CD4(+) T cells on the A3 domain of coagulation factor VIII. *J Thromb Haemost.* 2004;2(8):1385-1394.
41. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Human CD4+ T-cell epitope repertoire on the C2 domain of coagulation factor VIII. *J Thromb Haemost.* 2003;1(8):1777-1784.
42. James EA, Kwok WW, Ettinger RA, Thompson AR, Pratt KP. T-cell responses over time in a mild hemophilia A inhibitor subject: epitope identification and transient immunogenicity of the corresponding self-peptide. *J Thromb Haemost.* 2007;5(12):2399-2407.
43. James EA, van Haren SD, Ettinger RA, et al. T-cell responses in two unrelated hemophilia A inhibitor subjects include an epitope at the factor VIII R593C missense site. *J Thromb Haemost.* 2011;9(4):689-699.
44. Jacquemin M, Vantomme V, Buhot C, et al. CD4+ T-cell clones specific for wild-type factor VIII: a molecular mechanism responsible for a higher incidence of inhibitor formation in mild/moderate hemophilia A. *Blood.* 2003;101(4):1351-1358.
45. Madsen LS, Andersson EC, Jansson L, et al. A humanized model for multiple sclerosis using HLA-DR2 and a human T-cell receptor. *Nat Genet.* 1999; 23(3):343-347.
46. Pavlova A, Delev D, Lacroix-Desmazes S, et al. Impact of polymorphisms of the major histocompatibility complex class II, interleukin-10, tumor necrosis factor-alpha and cytotoxic T-lymphocyte antigen-4 genes on inhibitor development in severe hemophilia A. *J Thromb Haemost.* 2009;7(12):2006-2015.
47. Oldenburg J, Picard JK, Schwaab R, Brackmann HH, Tuddenham EG, Simpson E. HLA genotype of patients with severe haemophilia A due to intron 22 inversion with and without inhibitors of factor VIII. *Thromb Haemost.* 1997;77(2):238-242.
48. Hay CR, Ollier W, Pepper L, et al; UKHCDO Inhibitor Working Party. HLA class II profile: a weak determinant of factor VIII inhibitor development in severe haemophilia A. *Thromb Haemost.* 1997;77(2):234-237.
49. Cosgrove D, Gray D, Dierich A, et al. Mice lacking MHC class II molecules. *Cell.* 1991;66(5):1051-1066.
50. Toole JJ, Knopf JL, Wozney JM, et al. Molecular cloning of a cDNA encoding human antihaemophilic factor. *Nature.* 1984;312(5992):342-347.
51. Singh H, Raghava GP. ProPred: prediction of HLA-DR binding sites. *Bioinformatics.* 2001;17(12):1236-1237.
52. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanović S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics.* 1999;50(3-4):213-219.
53. Wang P, Sidney J, Dow C, Mothé B, Sette A, Peters B. A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. *PLOS Comput Biol.* 2008;4(4):e1000048.

54. Wang P, Sidney J, Kim Y, et al. Peptide binding predictions for HLA DR, DP and DQ molecules. *BMC Bioinformatics.* 2010;11(1):568.

55. Shen CR, Youssef AR, Devine A, et al. Peptides containing a dominant T-cell epitope from red cell band 3 have in vivo immunomodulatory properties in NZB mice with autoimmune hemolytic anemia. *Blood.* 2003;102(10):3800-3806.

56. Jansson L, Vrolix K, Jahraus A, Martin KF, Wraith DC. Immunotherapy with apitopes blocks the immune response to TSH receptor in HLA-DR transgenic mice. *Endocrinology.* 2018;159(9):3446-3457.

57. Anderton SM, Radu CG, Lowrey PA, Ward ES, Wraith DC. Negative selection during the peripheral immune response to antigen. *J Exp Med.* 2001; 193(1):1-11.

58. White J, Blackman M, Bill J, et al. Two better cell lines for making hybridomas expressing specific T cell receptors. *J Immunol.* 1989;143(6):1822-1825.

59. Fairchild PJ, Wildgoose R, Atherton E, Webb S, Wraith DC. An autoantigenic T cell epitope forms unstable complexes with class II MHC: a novel route for escape from tolerance induction. *Int Immunol.* 1993;5(9):1151-1158.

60. Mazza G, Ponsford M, Lowrey P, Campbell MJ, Zajicek J, Wraith DC. Diversity and dynamics of the T-cell response to MBP in DR2+ve individuals. *Clin Exp Immunol.* 2002;128(3):538-547.

61. Klyushnenkova EN, Kouiavskaia DV, Kodak JA, Vandenbark AA, Alexander RB. Identification of HLA-DRB1*1501-restricted T-cell epitopes from human prostatic acid phosphatase. *Prostate.* 2007;67(10):1019-1028.

62. Mitchison NA. Induction of immunological paralysis in two zones of dosage. *Proc R Soc Lond B Biol Sci.* 1964;161(983):275-292.

63. Kim YC, Zhang AH, Su Y, et al. Engineered antigen-specific human regulatory T cells: immunosuppression of FVIII-specific T- and B-cell responses. *Blood.* 2015;125(7):1107-1115.

64. van Haren SD, Herczenik E, ten Brinke A, et al. HLA-DR-presented peptide repertoires derived from human monocyte-derived dendritic cells pulsed with blood coagulation factor VIII. *Mol Cell Proteomics.* 2011;10(6):M110.002246.

65. Shepard ER, Wegner A, Hill EV, et al. The mechanism of action of antigen processing independent T cell epitopes designed for immunotherapy of autoimmune diseases. *Front Immunol.* 2021;12:654201.

66. Santambrogio L, Sato AK, Fischer FR, Dorf ME, Stern LJ. Abundant empty class II MHC molecules on the surface of immature dendritic cells. *Proc Natl Acad Sci USA.* 1999;96(26):15050-15055.

67. Venkatraman P, Nguyen TT, Sainlos M, et al. Fluorogenic probes for monitoring peptide binding to class II MHC proteins in living cells. *Nat Chem Biol.* 2007;3(4):222-228.

68. Hawiger D, Inaba K, Dorsett Y, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med.* 2001; 194(6):769-779.

69. Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol.* 2002;23(9): 445-449.

70. Reipert BM, Ahmad RU, Turecek PL, Schwarz HP. Characterization of antibodies induced by human factor VIII in a murine knockout model of hemophilia A. *Thromb Haemost.* 2000;84(5):826-832.

71. Whelan SF, Hofbauer CJ, Horling FM, et al. Distinct characteristics of antibody responses against factor VIII in healthy individuals and in different cohorts of hemophilia A patients. *Blood.* 2013;121(6):1039-1048.

72. Qadura M, Waters B, Burnett E, et al. Immunoglobulin isotypes and functional anti-FVIII antibodies in response to FVIII treatment in Balb/c and C57BL/6 haemophilia A mice. *Haemophilia.* 2011;17(2):288-295.

73. Hausl C, Maier E, Schwarz HP, et al. Long-term persistence of anti-factor VIII antibody-secreting cells in hemophilic mice after treatment with human factor VIII. *Thromb Haemost.* 2002;87(5):840-845.

74. Okamura T, Sumitomo S, Morita K, et al. TGF-β3-expressing CD4+CD25(-)LAG3+ regulatory T cells control humoral immune responses. *Nat Commun.* 2015;6(1):6329.

75. Lim HW, Hillsamer P, Banham AH, Kim CH. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *J Immunol.* 2005;175(7): 4180-4183.

76. Anderton SM, Wraith DC. Hierarchy in the ability of T cell epitopes to induce peripheral tolerance to antigens from myelin. *Eur J Immunol.* 1998;28(4): 1251-1261.

77. Campbell JD, Buckland KF, McMillan SJ, et al. Peptide immunotherapy in allergic asthma generates IL-10-dependent immunological tolerance associated with linked epitope suppression. *J Exp Med.* 2009;206(7):1535-1547.

78. Smith CE, Eagar TN, Strominger JL, Miller SD. Differential induction of IgE-mediated anaphylaxis after soluble vs. cell-bound tolerogenic peptide therapy of autoimmune encephalomyelitis. *Proc Natl Acad Sci USA.* 2005;102(27):9595-9600.